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TITLE: SURGICAL TREATMENT OF LASER INDUCED EYE INJURIES

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Subretinal blood within the macula may play a causative role in visual loss in a number of macular diseases. The clinical and histopathologic effects of experimental subretinal hemorrhage were evaluated in the cat. Subretinal hemorrhages were produced by creating a focal neurosensory retinal detachment with micropipette techniques, then inserting a needle tip transsclerally to allow choroidal blood to fill the bleb. Experimental lesions were examined clinically and with light and electron microscopy over a 14 day postoperative period. Initial observations included clot organization with retraction of fibrin strands tearing photoreceptor outer segments. Later degeneration progressed to involve all retinal layers overlying the clot. Hemorrhages into tissue plasminogen activator did not form fibrin strands nor cause photoreceptor tearing. These findings highlight the potential for improved retinal survival if organized subretinal clot can be eliminated soon after formation.

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Leonard Hjelmeland 12/5/90

PI Signature Date

Leonard M. Hjelmeland, Ph.D.

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INTRODUCTION

Aging macular degeneration is the leading cause of permanent blindness in people over age 60 in the industrialized world[1]. Subretinal hemorrhage from associated subretinal neovascular membranes may result in disciform macular scarring and permanent visual loss. Thus, there has been interest in the removal of subretinal hemorrhage to reduce the final disciform scar and the resultant area of visual loss[2-5]. The natural history and histopathology of retinal degeneration over subretinal hemorrhages remain poorly delineated to date. The mechanisms of injury may involve blood products, glial elements or neovascular membranes[6].

Studies of experimentally induced subretinal hemorrhages in animals have shown that blood alone can induce degenerative changes in the overlying retina[7-11]. These studies did not, however, establish the time course of damaging events associated with clot formation beneath the holangiotic (fully vascular) retina.

The present study demonstrates a model of subretinal hemorrhage which utilizes the holangiotic retina of the domestic cat and explores the natural history of early events following such a hemorrhage. The data suggest that fibrin directs the major mechanical injury and secondary degenerative processes in the first weeks after a subretinal hemorrhage.

MATERIALS AND METHODS

Study Design

In the study group (Table 1), subretinal hemorrhages were created beneath the retina adjacent to the area centralis in 10 domestic cats (Fig. 1). For a control group, focal neurosensory retinal detachments (blebs) without hemorrhages were created in five additional cats by the subretinal microinjection of balanced salt solution (BSS) with transvitreal micropipette techniques. Finally, in two other eyes (lesion 1t & 2t), tissue plasminogen activator (tPA, Genentech) was used to create the initial bleb into which the bleeding occurred. This created hemorrhages with fibrinolytic agents present prior to the hemorrhage and clot formation.

Animals

All animals were purchased from the Animal Resource Service of the University of California, Davis. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Animals were fed and watered ad libitum, and housed as a group with standard fluorescent lighting in twelve hour light-dark cycles. Laboratory studies included a complete blood count with differential, platelet estimate, prothrombin time, and a partial thromboplastin time. A dilated fundus exam was performed on all animals upon entry into the study.

Creation of subretinal lesions

All animals were premedicated with 0.5 mg/kg subcutaneous acepromazine maleate and 0.5 cc atropine sulfate, then placed under halothane general endotracheal anesthesia for creation of lesions. A lateral canthotomy was performed. A peritomy was created, and the four rectus muscles were isolated. Bipolar cautery was applied to maintain hemostasis. A pars plana sclerotomy was created 5.5 mm posterior to the limbus and enlarged to 1.5 mm with an MVR blade (Beaver). A limited core vitrectomy was performed with the Storz Microvit 1000 without infusion, to soften the eye. Subretinal blebs were created with a micropipette microinfusion system. A syringe pump (Harvard Apparatus) delivered fluid via IV extension tubing to an electrode coupler containing a glass micropipette which measured 40-50 μm outer diameter at the bevelled tip. A stereotactic micromanipulator holding the electrode coupler allowed controlled manipulation of the micropipette within the eye. The micropipette was advanced through the pars plana incision and across the vitreous cavity until it penetrated the neurosensory retina. Continuous volumetric pumping of the fluid at 5 μl/min for one minute was delivered beneath the retina to create a focal neurosensory retinal detachment. For control and experimental eyes with subretinal hemorrhages, BSS was delivered under the retina. For the tPA group, tPA solution at 200 μg/ml was used to create the bleb. To create an autologous subretinal hemorrhage, the tip of an MVR blade (Beaver Instruments) or a 25 gauge needle was passed transsclerally into the bleb and then withdrawn, allowing choroidal

blood to fill the bleb and extend under the retina into the area centralis. The sclerotomy was closed with vicryl suture and the lateral canthotomy with nylon suture. The eye then received subconjunctival injections of dexamethasone (2 mg) and gentamicin (20 mg) and topical applications of atropine and neomycin-dexamethasone.

Postoperative care

The animals were isolated for 8-16 hours after surgery. Topical atropine was applied BID and neomycin-dexamethasone TID for 7 days to the operated eye. Animals were followed with daily penlight examination. Slit lamp exam, indirect ophthalmoscopy and fundus photos were performed daily for 3 days and then every 2-4 days. For euthanasia, animals were premedicated with an intramuscular injection of ketamine hydrochloride (33 mg/kg), then given an intravenous injection of pentobarbital (85 mg/kg), followed by immediate enucleation.

Tissue Processing

Eyes were incised at the pars plana and placed in 2.5% glutaraldehyde, 1.5% paraformaldehyde with 0.1 M sodium cacodylate buffer at 4°C. After 15 minutes, the anterior segment including the lens was excised, and both segments were replaced in fixative. After 24 hours, the fixed posterior eyecups were sectioned, and specific lesions were photographed. Selected areas of tissue were taken for further microscopic analyses.

Light Microscopy

Tissues were dehydrated in graded ethanols and embedded in immuno-bed (Polysciences). Two to three micron sections were cut on a LKB Ultrotome III (Bromma). These were stained with Richardson's stain.

Transmission Electron Microscopy (TEM)

Tissues were post-fixed in osmium tetroxide, dehydrated in graded ethanols and embedded in Spurr's resin (Electron Microscopy Services). Sections were cut at 75 nm thickness, placed on 3 mm, 150 mesh copper grids, stained with lead citrate, and examined in a Phillips 410 transmission electron microscope.

Scanning Electron Microscopy (SEM)

Tissues were dehydrated in graded ethanols, critical point dried, and placed on aluminum mounts with Pelco colloidal silver paste (Ted Pella). These were sputter coated with gold and examined in a Phillips 501 scanning electron microscope

RESULTS

Clinical Results

Eleven subretinal hemorrhages were evaluated from 25 minutes through 14 days (Table 1,lesions 1-11). Very minimal anterior segment inflammation was noted on postoperative day one, which resolved within 24 hours. Due to bleeding from the pars plana sclerotomy site, 3 eyes had mild vitreous hemorrhage which settled

inferiorly within the first hour postoperatively. No vitreous organization was observed over the subretinal lesions. There was no evidence of subretinal blood leaking through the pipette track in any lesion.

The eleven subretinal hemorrhages were initially convex with a uniform, dense red appearance (Fig. 1). They developed a gravity oriented serum-erythrocyte meniscus within 3-6 hours after hemorrhage. In all lesions, there was a central area of organized coagulum which did not settle into a meniscus. Once formed, the meniscus and central organized lesion did not shift even with a change of head position for several hours. During the first 3 days, indirect ophthalmoscopy revealed retinal thickening, wrinkling and increased opacification observed over the central clot (Fig. 2). At 7 and 14 days, the wrinkling was less prominent and the retina appeared less edematous over the lesion.

Two additional hemorrhages were created into blebs containing tPA (lesion 1t and 2t). Clinically these appeared similar to the study hemorrhages. They did not enlarge or spread over the one hour of observation before enucleation.

The 5 eyes from the control group (BSS bleb with no hemorrhage) were followed for one hour, 7 days or 14 days. Very mild inflammation was noted in the anterior segment on postoperative day one. This resolved within 24 hours. The subretinal blebs were resorbed over 24 hours leaving an unremarkable clinical exam except for a small tapetal mark identifying the previous pipette entry site. The hematologic and

coagulation studies for all cats were within the normal ranges for our laboratory.

Histopathology Results

In the subretinal hemorrhages (lesions 1-11), clot organization with fibrin formation caused focal traction on photoreceptor outer segments (POS) with resultant mechanical retinal damage in the majority of lesions. Throughout the subretinal lesions, fibrin was identified morphologically and by its approximately 22.5 nm banding[12] pattern seen with TEM.

Within 25 minutes, fibrin was observed to interdigitate with the overlying photoreceptor layer (Fig. 3). By 1 hour, sheets of photoreceptor outer and inner segments were torn away from the overlying retina at regions of fibrin-POS interdigitation (Fig. 4). Neutrophils were prominent along the torn margins of the outer and inner segments. Except for this fibrin associated damage, the retina overlying the blood at one hour appeared essentially normal by light and electron microscopic examination. Within the subretinal space, the erythrocytes were packed centrally and bordered by the fibrin, platelets, leukocytes, and a rim of serous fluid.

At one day, the retina from the outer nuclear layer inward showed minimal degenerative changes over the blood. A gravity oriented differentiation of the subretinal clot was noted, with serous fluid present between the erythrocytes and the retina superiorly, while less fibrin and a denser packing of erythrocytes

was seen inferiorly. The clot appeared well organized, with macrophages, many leukocytes and fibrin marginated over the central area of erythrocytes. Channels of fibrin were identified crisscrossing the central areas of blood, and torn sheets of POS were seen displaced toward the center of the packed erythrocytes. A neutrophil response appeared prominent over the torn sections of POS and along the remaining disrupted margins of the vacuolating inner segments. By TEM, the photoreceptors appeared minimally vacuolated in areas where they remained intact. The inner retinal layers appeared morphologically unaffected by the underlying blood.

The lesions on day 2 and 3 demonstrated notable organization of the clot. This continued to appear gravity oriented with serum located superiorly, and erythrocytes packed inferiorly. The fibrin and leukocytic response was prominent at the erythrocyte serum meniscus, or at the superior margin of the erythrocytes when the serum had resorbed. In the two day lesion, in which the serous component had resorbed, large retinal folds were adherent to fibrin bands which may have been instrumental in their formation (Fig 5). This lesion demonstrated only very few small foci of fibrin associated photoreceptor tearing on serial sectioning. The intact photoreceptors showed vacuolization and degeneration. Both three day lesions demonstrated large sheets of torn photoreceptor outer segments adherent to fibrin bands in the organized area of clot (Fig. 6 and 7). Both hemorrhages showed some residual plasma superiorly, and one had prominent retinal folding. Phagocytic cells with ingested erythrocytes and photoreceptor outer segment

debris were present in all three lesions in fibrin areas and along the entire outer retinal surface. The neutrophil response was less prominent than at one day, particularly inferiorly in the lesions. Vacuolization of attached photoreceptor outer and inner segments was notable. The torn POS were more significantly deteriorated, with degenerating POS interspersed in areas of material no longer characteristic of POS (Fig. 8). There was minimal inner retinal vacuolization.

At 7 days, two separate retinal lesions in one eye demonstrated significant destruction of outer retinal elements over the blood. The erythrocytes remained densely packed, with fibrin throughout. The photoreceptor outer segments were torn from the retina over the entire surface of the clot in the larger lesion, with more severe degeneration of photoreceptor outer and inner segments into an amorphous band of lucent material (Fig 8). Minimal outer nuclear layer hypertrophy and loss occurred centrally. The inner retina demonstrated some increasing vacuolization. Macrophages were identified with ingested degenerated photoreceptor outer segments and erythrocytes. A fibrous/neovascular membrane was identified originating from the choroidal stab site within one clot. In both lesions, fibrous cells appeared to organize at the retina clot margins. Retinal pigment epithelial (RPE) cells showed shortened apical microvilli, mitochondrial distortion, rounding of the apical surface and reduplication centrally.

By day 14, there was extensive severe destruction of the outer retinal layers over significant portions of the hemorrhagic detachment (Fig. 9). Increasing numbers of phagocytic cells were observed in the dense erythrocyte layer and immediately overlying the RPE layer. In peripheral areas over the hemorrhage, vacuolization and shortening (Fig. 10). Superiorly where the serum had resorbed, there was minimal damage. In this area the retina resorbed, there was minimal damage. In this area the retina appeared similar to control reattached blebs. The greatest damage occurred where the most dense fibrin had organized at the upper edge of the erythrocyte meniscus. At this site,there was atrophy and disorganization of the outer retinal layers including the outer nuclear layer with proliferation of fibrocytic cells into this area. In addition, the inner nuclear layer showed significant vacuolization. Much less damage appeared in photoreceptors over packed erythrocytes inferiorly. Here the photoreceptor outer segments demonstrated minimal vacuolization which progressively worsened with POS atrophy as one moved upward toward the area of the erythrocyte-fibrin meniscus. Beneath the entire lesion, the RPE demonstrated confluent vacuoles, disorganization of cytoplasm, and reduplication which was more prominent centrally where it was associated with overlying fibrocytic cells.

In the 1 hour hemorrhages into the PPA solution (lesions 1t and 2t), there was extremely minimal fibrin formation with no fibrin band formation. The overlying retina, including photoreceptor outer segments, was intact.

Evaluation of the RPE base and neurosensory retina overlying the control BSS blebs by SEM demonstrated minimal pathology from simple bleb formation. Fibrin strands coated the RPE, but very few individual torn POS were identified. Serial sections with light and TEM evaluation of these blebs showed no significant morphological alterations. The control lesions demonstrated few focal structural changes in the RPE and photoreceptors which have been reported by others in studies of experimental retinal detachment and reattachment[13-16]. Such findings included: at one hour, apical mounding of RPE cells; at 7 days, minimal POS shortening and irregularity and a few sites of RPE reduplication in focal residual microdetachments of less than 20 μm. At 14 days, no photoreceptor degeneration or atrophy was identified in the reattached BSS lesions.

DISCUSSION

Previous studies of subretinal hemorrhage have either used a merangiotic (partially vascularized) retinal model, or have not investigated the progression of clot organization in the first 14 days. Koshibu[7-9] described the degradation and resorption of erythrocytes over 6 months in the rat eye following the subretinal injection of a saline-blood-heparin mixture. He observed photoreceptor outer segment disruption after two days, and inner segment degeneration with pyknotic nuclei at 20 days after blood injection. Because of heparinization, this study did not address the issue of the organization of blood elements and the effects of

clot formation on survival of the overlying retina. Glatt and Machemer[10] reported irreversible retinal degeneration in their rabbit model of subretinal hemorrhage within 24 hours. A notable finding was that the degeneration was more marked in nonvascularized retinal areas over the hemorrhage. Their examination of subretinal blood in a single cat at three days suggested less retinal degeneration than in the rabbit.

In developing the technique to create subretinal hemorrhage in this study, the transscleral technique of Glatt and Machemer was selected and combined with classic subretinal bleb formation to ensure minimal retinal damage in creation of these lesions. Utilizing the transscleral hemorrhage technique, a significant autologous hemorrhage could be consistently produced. Maintaining a low intraocular pressure at the time of choroidal subretinal bleeding was an important factor in ensuring significant subretinal hemorrhages. The pipette technique, with a low flow of volumetric pumping, induced no significant mechanical injury to the neurosensory retina or RPE during bleb formation. Only focal injury along the pipette track was identified. This was verified by light microscopy, TEM and SEM of fresh subretinal blebs. With this technique, there was no leakage of subretinal blood through the retinal pipette hole. This was consistent with the observation of Marmor[17], who demonstrated that with a micropipette tip of 40-50 μm, one or more pipette tracks had no influence on outflow of materials from a subretinal bleb.

This study was undertaken to determine the early events associated with subretinal hemorrhage in a holangiotic model. The most striking finding in the first hour after hemorrhage was rapid formation of a fibrin clot. The density of the fibrin meshwork over and throughout the clot, though variable, was consistently associated with fibrin interdigitation with the photoreceptors. The fibrin appeared to cause the most significant early pathology due to mechanical shearing of the photoreceptors. In contrast, this mechanical damage was absent in the relatively fibrin free hemorrhages in which tPA was present at formation.

By comparison, in the rabbit model of hemorrhage beneath merangiotic retina, Glatt and Machemer[10] noted fibrin within the subretinal blood but only minimal retinal changes (edematous photoreceptors) 1 hour after hemorrhage. Perhaps the cat inflammatory or platelet response is significantly different from the rabbit, or the holangiotic retina responds more rapidly to induce significant fibrin organization.

Fibrin effects also direct the areas of degeneration in later lesions. Retinal areas containing torn photoreceptors appeared to develop the most severe degeneration within the 14 day time frame. This may be due to simple mechanical damage of the cell layer, or because of an immediate inflammatory cell response with phagocytosis of cell remnants and production of inflammatory "toxins". The fibrin-dense areas, oriented at the meniscus between the erythrocytes and plasma, appear to be the sites of severe, outer retinal degeneration. Less retinal degeneration appeared

above and below this area. Indeed, the retina over densely packed erythrocytes in the inferior areas of the hemorrhage demonstrated very minimal histopathologic changes. This suggests that it is not the mere presence of erythrocytes as a barrier which causes the early degeneration, but rather fibrin and perhaps other inflammatory products at the sedimentation junction, which are responsible for retinal injury.

An interesting finding is the ingrowth of fibrocytic cells in all seven and fourteen day eyes, and a single large organized fibrovascular membrane at the seven day time period. Ryan created similar transscleral subretinal hemorrhages while trying to produce subretinal neovascular membranes[11]. The neovascular membranes were not consistently produced in his model.

The present study demonstrates a model of hemorrhage beneath the holangiotic retina of the domestic cat. Identified within this model is a sequence of clot organization beneath the retina, associated retinal damage and sites of progressive retinal degeneration during the first weeks after injury. Progressive severe injury occurs over 7-14 days after hemorrhage, in some overlying retina.

Early damage from subretinal hemorrhage with fibrin formation might be a consideration in a human patient with an intraoperative subretinal hemorrhage. Removal of the blood prior to the establishment of a fibrin scaffold could be an important aspect of visual recovery. The fibrin adhesions identified throughout the first 7 days in the cat model make surgical

removal of such a subretinal clot inadvisable without the preceding use of a fibrinolytic agent.

Clinical exam of the retina over the clots revealed nc difference in the appearance of lesions which had photoreceptor tearing when compared to those in which no tearing occurred. In a patient it may be similarly difficult to identify whether photoreceptor tearing has occurred, or how extensive this may be. If large photoreceptor areas should be torn in a human patient, the retina may do poorly despite fibrinolytic injection or blood removal. The time interval for any attempted subretinal surgery would appear to be prior to the onset of irreversible damage, which would be before seven days in the domestic cat.

This model could be useful for further studies of the cellular events involved in the degeneration of the retina over a clot. The results suggest that fibrin involvement in retinal damage should be more critically examined in the early interval after subretinal hemorrhage, and that fibrin may be an important consideration in planning treatment or removal of such a subretinal hemorrhage.

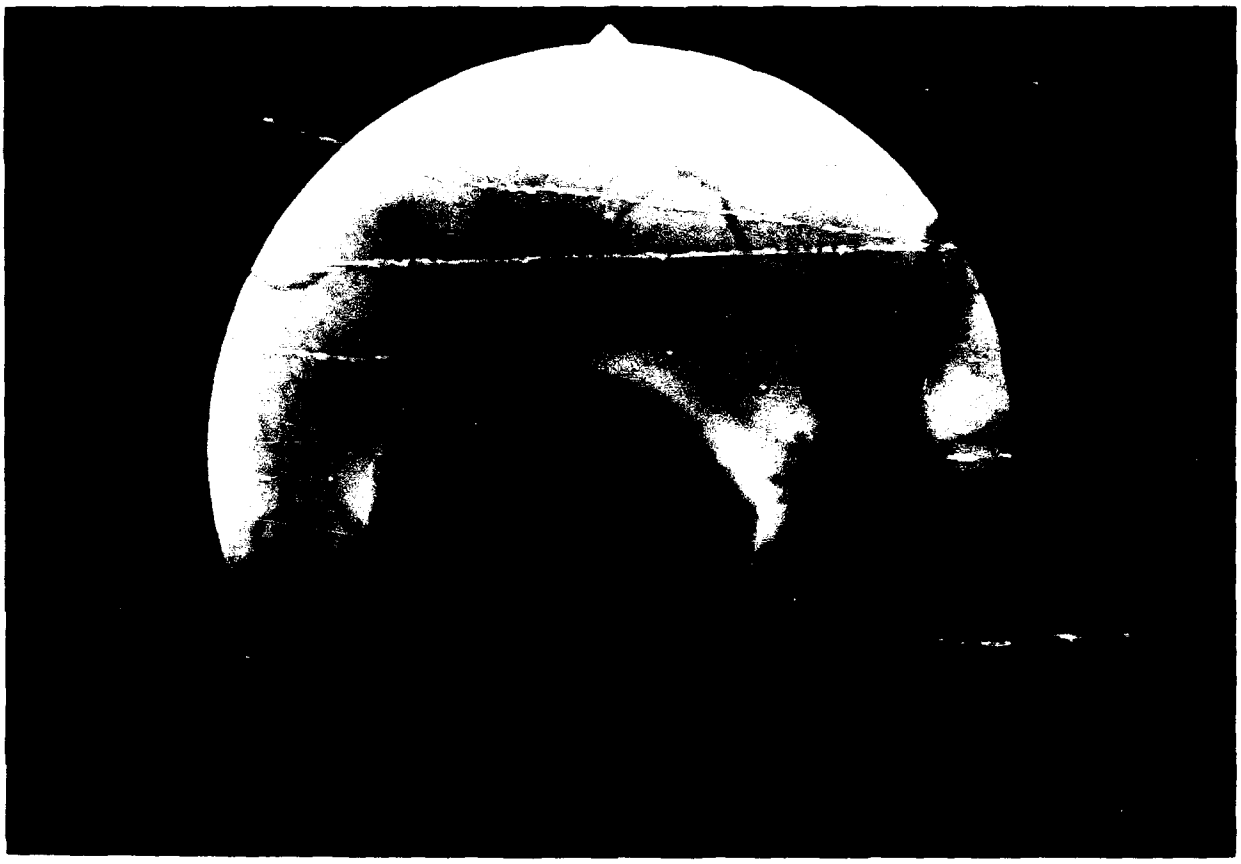

Figure 1

Fundus photograph of a one hour old subretinal hemorrhage in the area centralis (lesion 11).

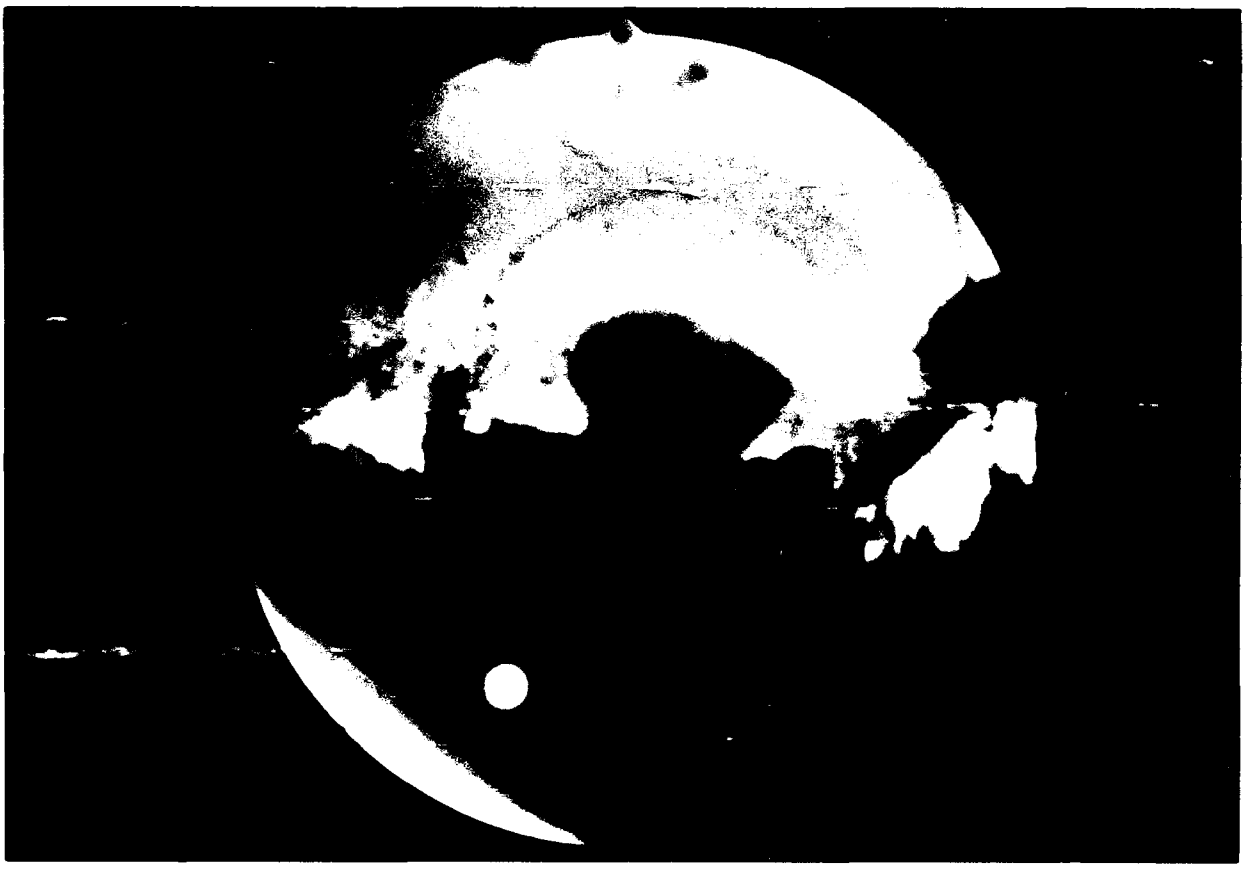

Figure 2

Fundus photograph of a 3 day old subretinal hemorrhage (lesion 11). A serum-erythrocyte meniscus is evident along with a central area of organized coagulum. Retinal wrinkling and opacification can be seen over the central clot.

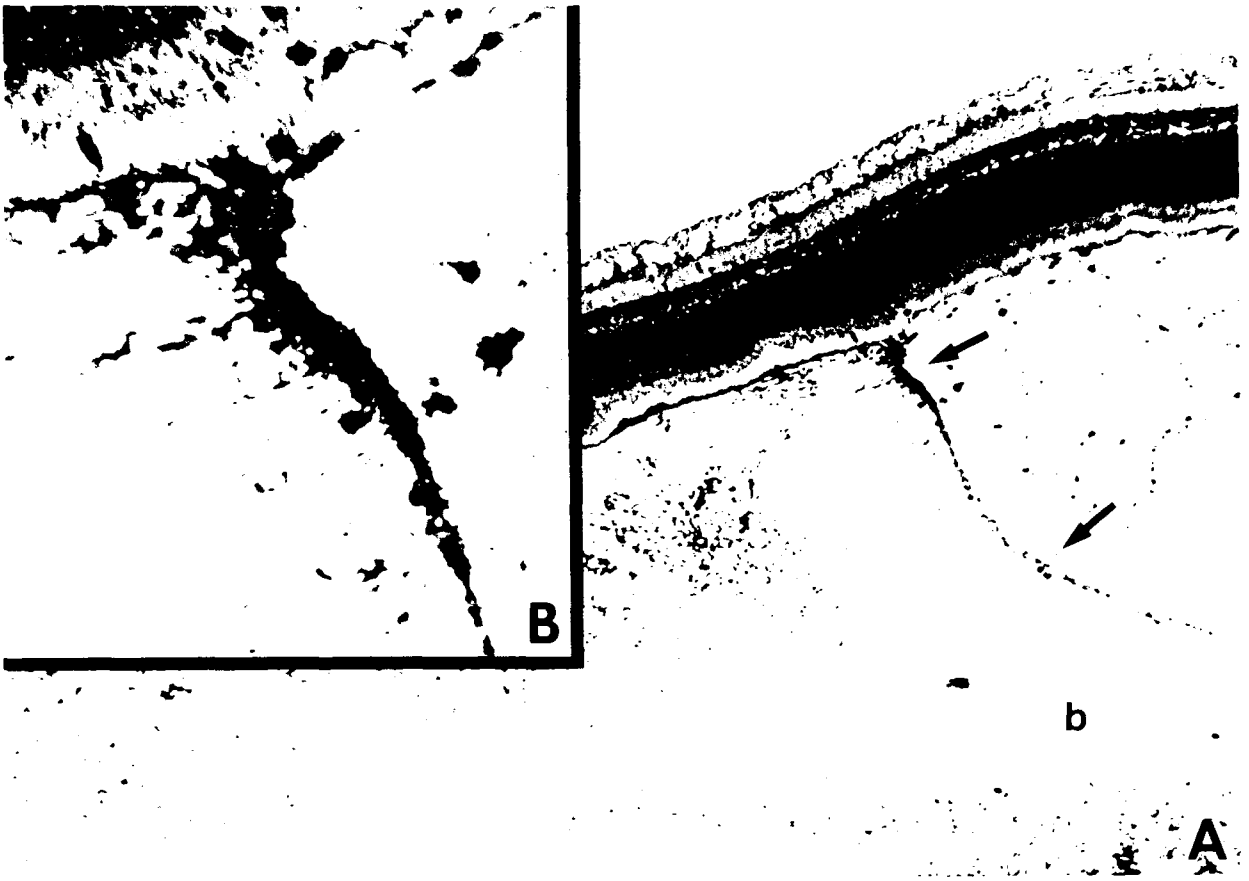

Figure 3

Figure 3a : Fibrin (arrows) in a 25 minute old subretinal hemorrhage (lesion 1) is identified in organized strands extending through the subretinal blood (b) to end in a dense attachment at the photoreceptor outer segments (Richardsons stain). Magnification = 125X

Figure 3b (inset) : Note the area of attachment of the fibrin strand to the photoreceptor outer segments, and the presence of neutrophils along the organized fibrin strand. Magnification = 500X

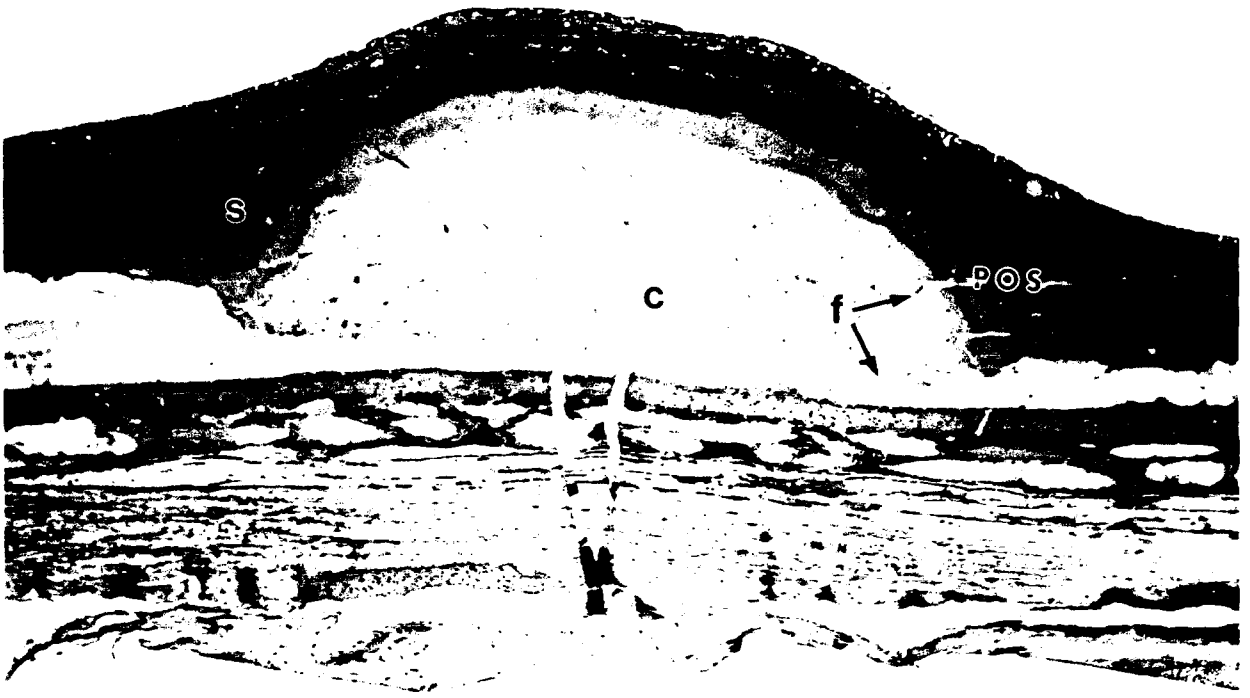

Figure 4

Clot organization in a 1 hour old subretinal hemorrhage (lesion 4). The clot (c) is densely packed centrally with criscrossing fibrin strands (f). Marginated over the central clot is a dense layer of fibrin with torn photoreceptor outer segments adherent in a sheet to the clot's surface. The serum component (s) separates this from the remaining retinal layers (Richardsons stain). Magnification = 50X

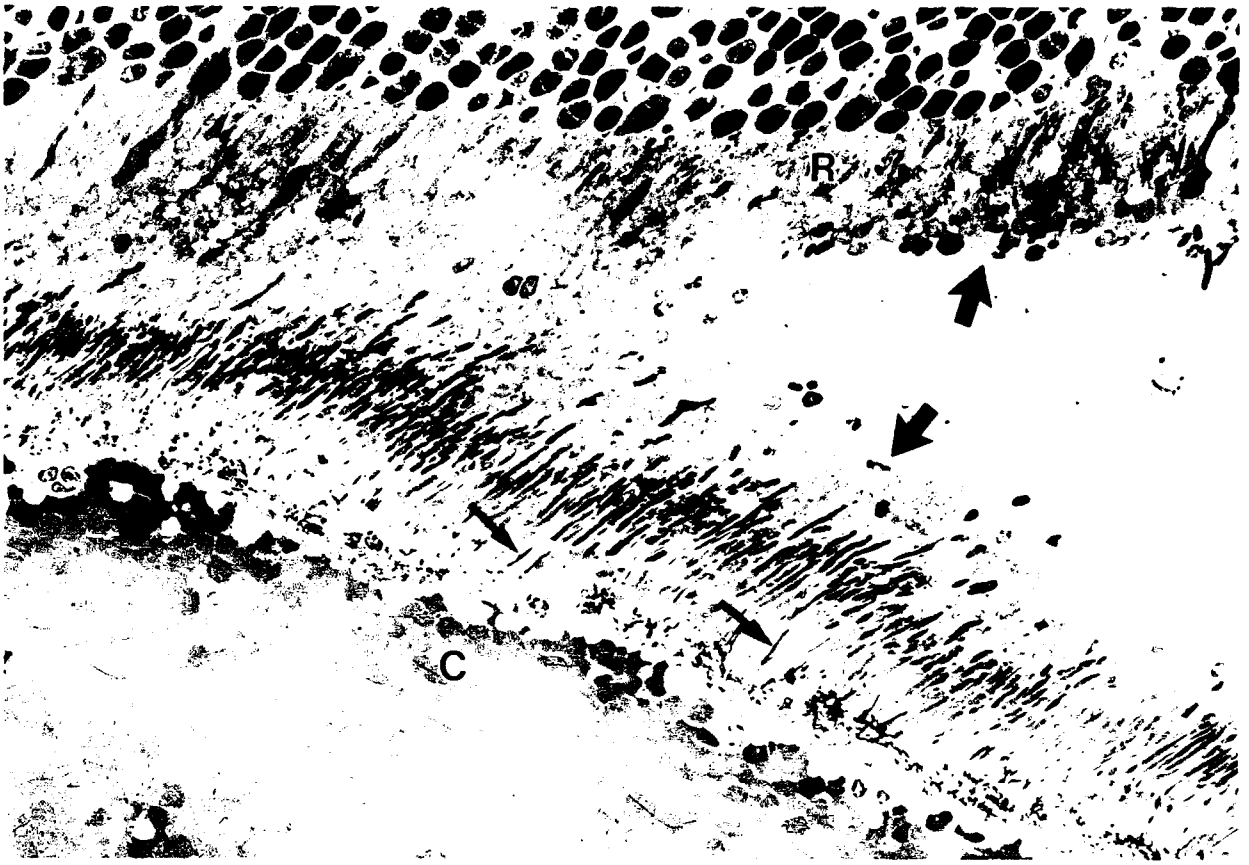

Figure 5

Transmission electron micrograph of a 1 hour old subretinal hemorrhage (lesion 4). This area of the clot demonstrates erythrocytes (e) bordered by a layer of lymphocytes, platelets, and fibrin. Note the fibrin (arrows) interdigitating with the photoreceptor outer segments. Wide arrows indicate torn surfaces where photoreceptor inner and outer segments separated from the remaining retinal layers (r). Magnification = 1400X

Figure 6

A two day old subretinal hemorrhage (lesion 6) demonstrating retinal folds adherent to fibrin bands (arrows) which criscross the packed erythrocytes (e) (Richardsons stain). Magnification = 50X

Figure 7

A 3 day old subretinal hemorrhage (lesion 7) demonstrating the gravity oriented clot organization and sheets of torn, displaced photoreceptor outer segments (Richardsons stain). The superior margin of the lesion is marked (*). In this area the serum has resorbed. The densest area of fibrin orgnaization (arrows) is located at the erythrocyte serum meniscus. More inferiorly in this lesion, packed erythrocytes (e) with less fibrin correspond to the settled erythrocytes as seen in a typical clinical picture (Fig. 2). Magnification = 50X

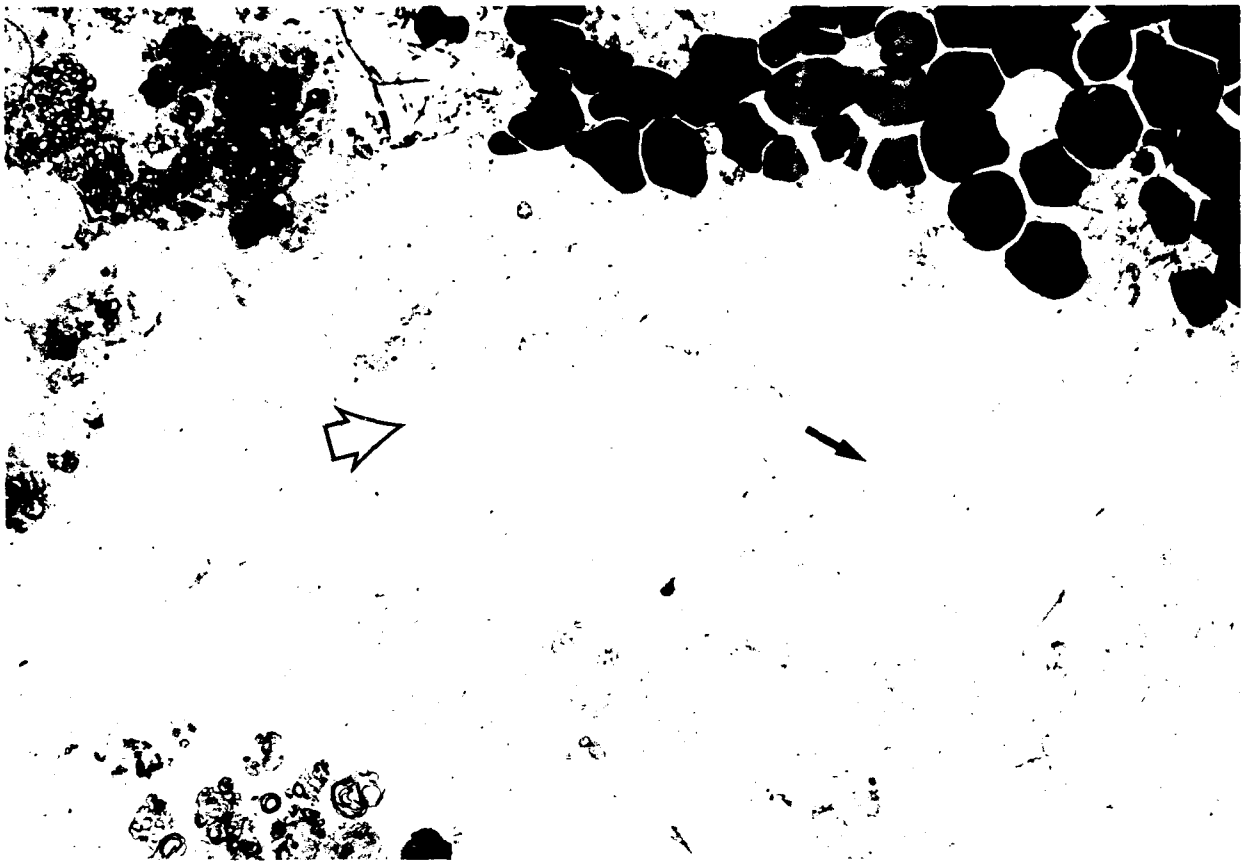

Figure 8

Transmission electron micrograph of a section of torn photoreceptor outer segments (open arrows) which were displaced centrally in the 3 day old clot (lesion 7). The segments still interdigitate with a meshwork of fibrin (f). Note the severe degeneration of photoreceptor outer segments with loss of identifiable cellular elements. Magnification = 2,650X

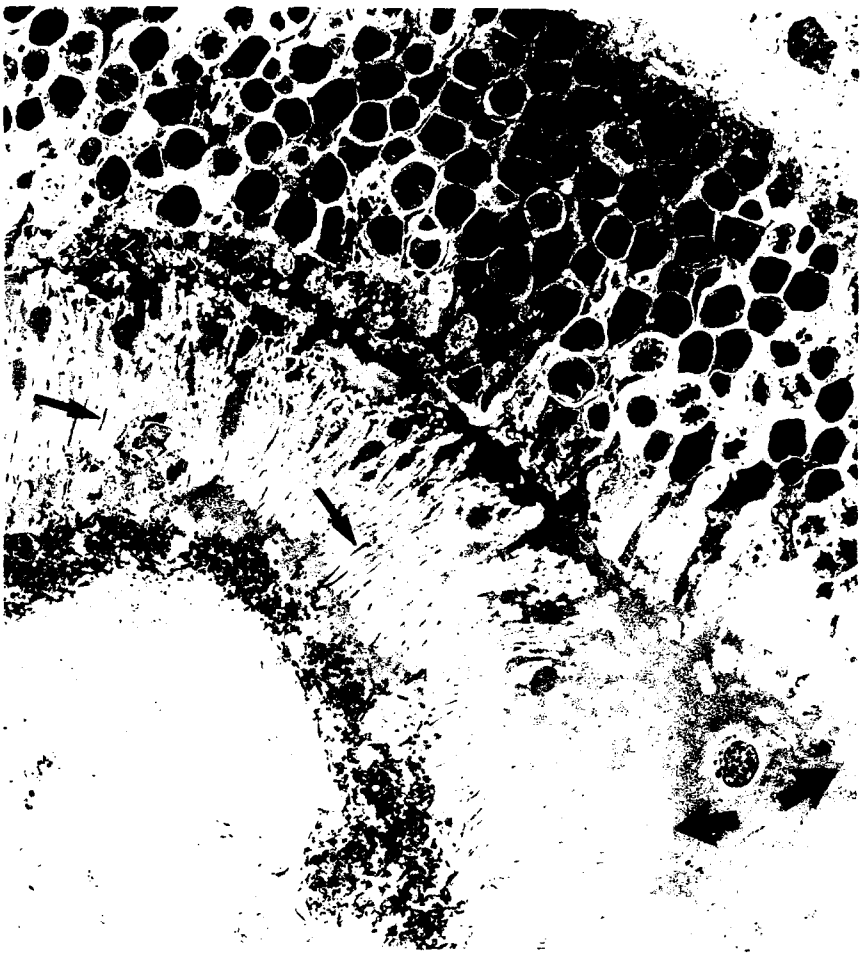

Figure 9

Transmission electron micrograph of an area of 7 day old subretinal hemorrhage (lesion 10). Fibrin (small arrows) is identified interdigitating with amorphous material which resembles degenerated outer segment material (seen in Figure 7). The photoreceptor outer segments are separated in several areas from the overlying retinal layers (open arrows). The photoreceptor inner segments also show degeneration. Magnification = 1,300X

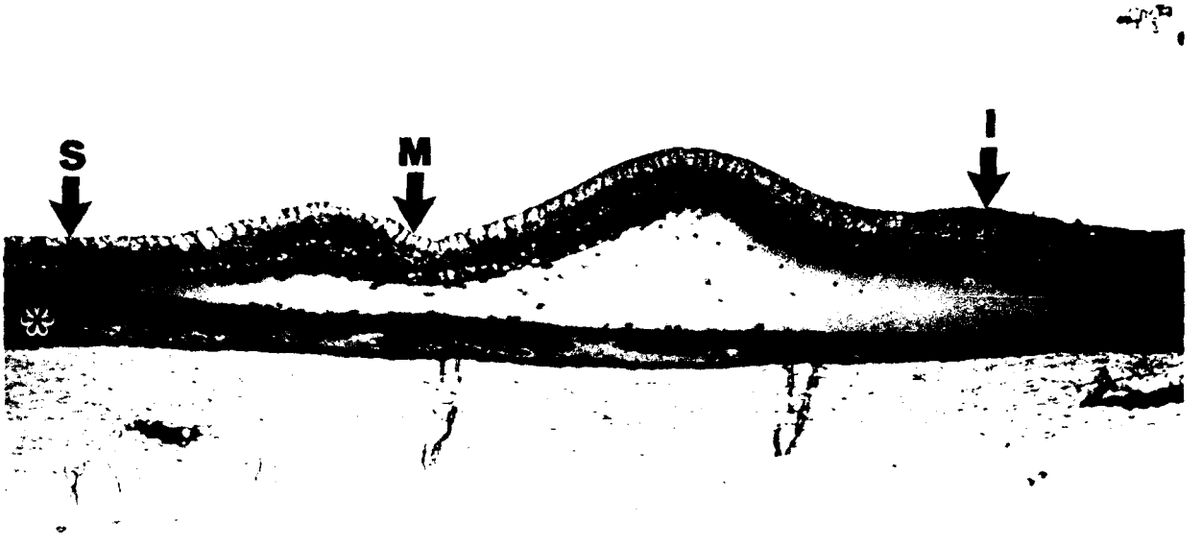

Figure 10

14 day old subretinal hemorrhage (lesion 11) with localization of retinal degeneration over sites near the serum-erythrocyte meniscus (Richardsons stain). The superior margin of the lesion is marked (*). The entire lesion is not in the photograph. Minimal retinal damage is seen superiorly where the serum had resorbed (s) and inferiorly over the packed erythrocytes (i). The retina overlying areas of the serum-erythrocyte meniscus (m) demonstrates loss and disorganization of outer retinal layers. Magnification = 50X

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